

## NOVEL APPROACH TO QUANTITATIVE POLYMERASE CHAIN REACTION USING REAL-TIME DETECTION: APPLICATION TO THE DETECTION OF GENE AMPLIFICATION IN BREAST CANCER

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Gene amplification is a common event in the progression of human cancers, and amplified oncogenes have been shown to have diagnostic, prognostic and therapeutic relevance. A kinetic quantitative polymerase-chain-reaction (PCR) method, based on fluorescent TaqMan methodology and a new instrument (ABI Prism 7700 Sequence Detection System) capable of measuring fluorescence in real-time, was used to quantify gene amplification in tumor DNA. Reactions are characterized by the point during cycling when PCR amplification is still in the exponential phase, rather than the amount of PCR product accumulated after a fixed number of cycles. None of the reaction components is limited during the exponential phase, meaning that values are highly reproducible in reactions starting with the same copy number. This greatly improves the precision of DNA quantification. Moreover, real-time PCR does not require post-PCR sample handling, thereby preventing potential PCR-product carry-over contamination; it possesses a wide dynamic range of quantification and results in much faster and higher sample throughput. The real-time PCR method, was used to develop and validate a simple and rapid assay for the detection and quantification of the 3 most frequently amplified genes (*myc*, *ccnd1* and *erbB2*) in breast tumors. Extra copies of *myc*, *ccnd1* and *erbB2* were observed in 10, 23 and 15%, respectively, of 108 breast-tumor DNA; the largest observed numbers of gene copies were 4.6, 18.6 and 15.1, respectively. These results correlated well with those of Southern blotting. The use of this new semi-automated technique will make molecular analysis of human cancers simpler and more reliable, and should find broad applications in clinical and research settings. *Int. J. Cancer* 78:661–666, 1998.

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Gene amplification plays an important role in the pathogenesis of various solid tumors, including breast cancer, probably because over-expression of the amplified target genes confers a selective advantage. The first technique used to detect genomic amplification was cytogenetic analysis. Amplification of several chromosome regions, visualized either as extrachromosomal double minutes (dmns) or as integrated homogeneously staining regions (HSRs), are among the main visible cytogenetic abnormalities in breast tumors. Other techniques such as comparative genomic hybridization (CGH) (Kallioniemi *et al.*, 1994) have also been used in broad searches for regions of increased DNA copy numbers in tumor cells, and have revealed some 20 amplified chromosome regions in breast tumors. Positional cloning efforts are underway to identify the critical gene(s) in each amplified region. To date, genes known to be amplified frequently in breast cancers include *myc* (8q24), *ccnd1* (11q13), and *erbB2* (17q12-q21) (for review, see Bièche and Lidereau, 1995).

Amplification of the *myc*, *ccnd1*, and *erbB2* proto-oncogenes should have clinical relevance in breast cancer, since independent studies have shown that these alterations can be used to identify sub-populations with a worse prognosis (Berns *et al.*, 1992; Schuurin *et al.*, 1992; Slamon *et al.*, 1987). Muss *et al.* (1994) suggested that these gene alterations may also be useful for the prediction and assessment of the efficacy of adjuvant chemotherapy and hormone therapy.

However, published results diverge both in terms of the frequency of these alterations and their clinical value. For instance, over 500 studies in 10 years have failed to resolve the controversy

surrounding the link suggested by Slamon *et al.* (1987) between *erbB2* amplification and disease progression. These discrepancies are partly due to the clinical, histological and ethnic heterogeneity of breast cancer, but technical considerations are also probably involved.

Specific genes (DNA) were initially quantified in tumor cells by means of blotting procedures such as Southern and slot blotting. These batch techniques require large amounts of DNA (5–10 µg/reaction) to yield reliable quantitative results. Furthermore, meticulous care is required at all stages of the procedures to generate blots of sufficient quality for reliable dosage analysis. Recently, PCR has proven to be a powerful tool for quantitative DNA analysis, especially with minimal starting quantities of tumor samples (small, early-stage tumors and formalin-fixed, paraffin-embedded tissues).

Quantitative PCR can be performed by evaluating the amount of product either after a given number of cycles (end-point quantitative PCR) or after a varying number of cycles during the exponential phase (kinetic quantitative PCR). In the first case, an internal standard distinct from the target molecule is required to ascertain PCR efficiency. The method is relatively easy but implies generating, quantifying and storing an internal standard for each gene studied. Nevertheless, it is the most frequently applied method to date.

One of the major advantages of the kinetic method is its rapidity in quantifying a new gene, since no internal standard is required (an external standard curve is sufficient). Moreover, the kinetic method has a wide dynamic range (at least 5 orders of magnitude), giving an accurate value for samples differing in their copy number. Unfortunately, the method is cumbersome and has therefore been rarely used. It involves aliquot sampling of each assay mix at regular intervals and quantifying, for each aliquot, the amplification product. Interest in the kinetic method has been stimulated by a novel approach using fluorescent TaqMan methodology and a new instrument (ABI Prism 7700 Sequence Detection System) capable of measuring fluorescence in real time (Gibson *et al.*, 1996; Heid *et al.*, 1996). The TaqMan reaction is based on the 5' nuclease assay first described by Holland *et al.* (1991). The latter uses the 5' nuclease activity of Taq polymerase to cleave a specific fluorogenic oligonucleotide probe during the extension phase of PCR. The approach uses dual-labeled fluorogenic hybridization probes (Lee *et al.*, 1993). One fluorescent dye, co-valently linked to the 5' end of the oligonucleotide, serves as a reporter [FAM (*i.e.*, 6-carboxy-fluorescein)] and its emission spectrum is quenched by a second fluorescent dye, TAMRA (*i.e.*, 6-carboxy-tetramethyl-rhodamine) attached to the 3' end. During the extension phase of the PCR

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cycle, the fluorescent hybridization probe is hydrolyzed by the 5'-3' nucleolytic activity of DNA polymerase. Nuclease degradation of the probe releases the quenching of FAM fluorescence emission, resulting in an increase in peak fluorescence emission. The fluorescence signal is normalized by dividing the emission intensity of the reporter dye (FAM) by the emission intensity of a reference dye (i.e., ROX, 6-carboxy-X-rhodamine) included in TaqMan buffer, to obtain a ratio defined as the  $R_n$  (normalized reporter) for a given reaction tube. The use of a sequence detector enables the fluorescence spectra of all 96 wells of the thermal cycler to be measured continuously during PCR amplification.

The real-time PCR method offers several advantages over other current quantitative PCR methods (Celi *et al.*, 1994): (i) the probe-based homogeneous assay provides a real-time method for detecting only specific amplification products, since specific hybridization of both the primers and the probe is necessary to generate a signal; (ii) the  $C_t$  (threshold cycle) value used for quantification is measured when PCR amplification is still in the log phase of PCR product accumulation. This is the main reason why  $C_t$  is a more reliable measure of the starting copy number than are end-point measurements, in which a slight difference in a limiting component can have a drastic effect on the amount of product; (iii) use of  $C_t$  values gives a wider dynamic range (at least 5 orders of magnitude), reducing the need for serial dilution; (iv) The real-time PCR method is run in a closed-tube system and requires no post-PCR sample handling, thus avoiding potential contamination; (v) the system is highly automated, since the instrument continuously measures fluorescence in all 96 wells of the thermal cycler during PCR amplification and the corresponding software processes, and analyzes the fluorescence data; (vi) the assay is rapid, as results are available just one minute after thermal cycling is complete; (vii) the sample throughput of the method is high, since 96 reactions can be analyzed in 2 hr.

Here, we applied this semi-automated procedure to determine the copy numbers of the 3 most frequently amplified genes in breast tumors (*myc*, *ccnd1* and *erbB2*), as well as 2 genes (*alb* and *app*) located in a chromosome region in which no genetic changes have been observed in breast tumors. The results for 108 breast tumors were compared with previous Southern-blot data for the same samples.

#### MATERIAL AND METHODS

##### Tumor and blood samples

Samples were obtained from 108 primary breast tumors removed surgically from patients at the Centre René Huguenin; none of the patients had undergone radiotherapy or chemotherapy. Immediately after surgery, the tumor samples were placed in liquid nitrogen until extraction of high-molecular-weight DNA. Patients were included in this study if the tumor sample used for DNA preparation contained more than 60% of tumor cells (histological analysis). A blood sample was also taken from 18 of the same patients.

DNA was extracted from tumor tissue and blood leukocytes according to standard methods.

##### Real-time PCR

**Theoretical basis.** Reactions are characterized by the point during cycling when amplification of the PCR product is first detected, rather than by the amount of PCR product accumulated after a fixed number of cycles. The higher the starting copy number of the genomic DNA target, the earlier a significant increase in fluorescence is observed. The parameter  $C_t$  (threshold cycle) is defined as the fractional cycle number at which the fluorescence generated by cleavage of the probe passes a fixed threshold above baseline. The target gene copy number in unknown samples is quantified by measuring  $C_t$  and by using a standard curve to determine the starting copy number. The precise amount of genomic DNA (based on optical density) and its quality (i.e., lack

of extensive degradation) are both difficult to assess. We therefore also quantified a control gene (*alb*) mapping to chromosome region 4q11-q13, in which no genetic alterations have been found in breast-tumor DNA by means of CGH (Kallioniemi *et al.*, 1994).

Thus, the ratio of the copy number of the target gene to the copy number of the *alb* gene normalizes the amount and quality of genomic DNA. The ratio defining the level of amplification is termed "N", and is determined as follows:

$$N = \frac{\text{copy number of target gene (app, myc, ccnd1, erbB2)}}{\text{copy number of reference gene (alb)}}$$

**Primers, probes, reference human genomic DNA and PCR consumables.** Primers and probes were chosen with the assistance of the computer programs Oligo 4.0 (National Biosciences, Plymouth, MN), EuGene (Daniben Systems, Cincinnati, OH) and Primer Express (Perkin-Elmer Applied Biosystems, Foster City, CA).

Primers were purchased from DNAgency (Malvern, PA) and probes from Perkin-Elmer Applied Biosystems.

Nucleotide sequences for the oligonucleotide hybridization probes and primers are available on request.

The TaqMan PCR Core reagent kit, MicroAmp optical tubes, and MicroAmp caps were from Perkin-Elmer Applied Biosystems.

**Standard-curve construction.** The kinetic method requires a standard curve. The latter was constructed with serial dilutions of specific PCR products, according to Piatak *et al.* (1993). In practice, each specific PCR product was obtained by amplifying 20 ng of a standard human genomic DNA (Boehringer, Mannheim, Germany) with the same primer pairs as those used later for real-time quantitative PCR. The 5 PCR products were purified using MicroSpin S-400 HR columns (Pharmacia, Uppsala, Sweden) electrophoresed through an acrylamide gel and stained with ethidium bromide to check their quality. The PCR products were then quantified spectrophotometrically and pooled, and serially diluted 10-fold in mouse genomic DNA (Clontech, Palo Alto, CA) at a constant concentration of 2 ng/ $\mu$ l. The standard curve used for real-time quantitative PCR was based on serial dilutions of the pool of PCR products ranging from  $10^{-7}$  ( $10^5$  copies of each gene) to  $10^{-10}$  ( $10^2$  copies). This series of diluted PCR products was aliquoted and stored at  $-80^\circ\text{C}$  until use.

The standard curve was validated by analyzing 2 known quantities of calibrator human genomic DNA (20 ng and 50 ng).

**PCR amplification.** Amplification mixes (50  $\mu$ l) contained the sample DNA (around 20 ng, around 6600 copies of disomic genes), 10 $\times$  TaqMan buffer (5  $\mu$ l), 200  $\mu$ M dATP, dCTP, dGTP, and 400  $\mu$ M dUTP, 5 mM MgCl<sub>2</sub>, 1.25 units of AmpliTaq Gold, 0.5 units of AmpErase uracil N-glycosylase (UNG), 200 nM each primer and 100 nM probe. The thermal cycling conditions comprised 2 min at  $50^\circ\text{C}$  and 10 min at  $95^\circ\text{C}$ . Thermal cycling consisted of 40 cycles at  $95^\circ\text{C}$  for 15 s and  $65^\circ\text{C}$  for 1 min. Each assay included: a standard curve (from  $10^5$  to  $10^2$  copies) in duplicate, a no-template control, 20 ng and 50 ng of calibrator human genomic DNA (Boehringer) in triplicate, and about 20 ng of unknown genomic DNA in triplicate (26 samples can thus be analyzed on a 96-well microplate). All samples with a coefficient of variation (CV) higher than 10% were retested.

All reactions were performed in the ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems), which detects the signal from the fluorogenic probe during PCR.

**Equipment for real-time detection.** The 7700 system has a built-in thermal cycler and a laser directed via fiber optical cables to each of the 96 sample wells. A charge-coupled-device (CDD) camera collects the emission from each sample and the data are analyzed automatically. The software accompanying the 7700 system calculates  $C_t$  and determines the starting copy number in the samples.

**Determination of gene amplification.** Gene amplification was calculated as described above. Only samples with an  $N$  value higher than 2 were considered to be amplified.

### RESULTS

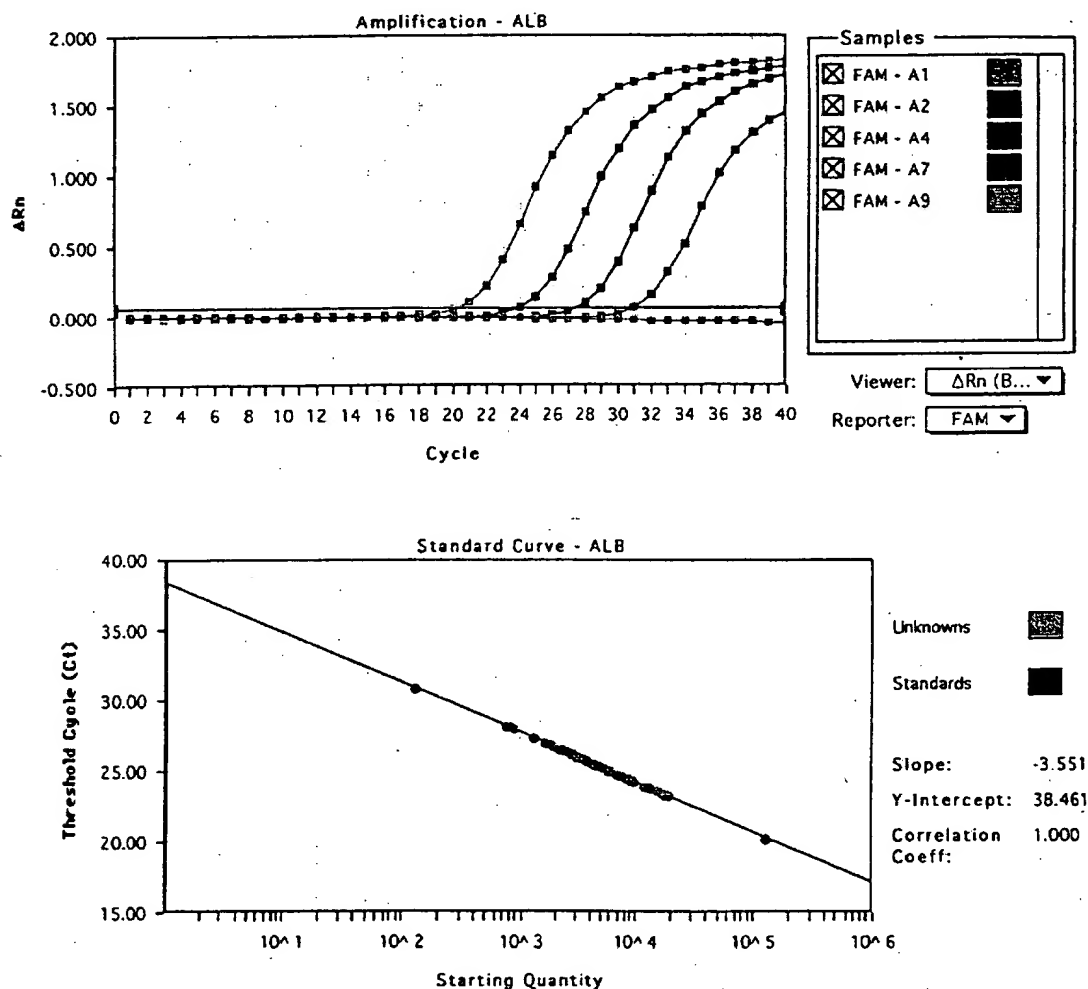
To validate the method, real-time PCR was performed on genomic DNA extracted from 108 primary breast tumors, and 18 normal leukocyte DNA samples from some of the same patients. The target genes were the *myc*, *ccnd1*, and *erbB2* proto-oncogenes, and the  $\beta$ -amyloid precursor protein gene (*app*), which maps to a chromosome region (21q21.2) in which no genetic alterations have been found in breast tumors (Kallioniemi *et al.*, 1994). The reference disomic gene was the albumin gene (*alb*, chromosome 4q11-q13).

### Validation of the standard curve and dynamic range of real-time PCR

The standard curve was constructed from PCR products serially diluted in genomic mouse DNA at a constant concentration of 2 ng/ $\mu$ l. It should be noted that the 5 primer pairs chosen to analyze the 5 target genes do not amplify genomic mouse DNA (data not shown). Figure 1 shows the real-time PCR standard curve for the *alb* gene. The dynamic range was wide (at least 4 orders of magnitude), with samples containing as few as  $10^2$  copies or as many as  $10^5$  copies.

### Copy-number ratio of the 2 reference genes (*app* and *alb*)

The *app* to *alb* copy-number ratio was determined in 18 normal leukocyte DNA samples and all 108 primary breast-tumor DNA



**FIGURE 1** – Albumin (*alb*) gene dosage by real-time PCR. Top: Amplification plots for reactions with starting *alb* gene copy number ranging from  $10^5$  (A9),  $10^4$  (A7),  $10^3$  (A4) to  $10^2$  (A2) and a no-template control (A1). Cycle number is plotted vs. change in normalized reporter signal ( $\Delta Rn$ ). For each reaction tube, the fluorescence signal of the reporter dye (FAM) is divided by the fluorescence signal of the passive reference dye (ROX), to obtain a ratio defined as the normalized reporter signal ( $Rn$ ).  $\Delta Rn$  represents the normalized reporter signal ( $Rn$ ) minus the baseline signal established in the first 15 PCR cycles.  $\Delta Rn$  increases during PCR as *alb* PCR product copy number increases until the reaction reaches a plateau.  $C_t$  (threshold cycle) represents the fractional cycle number at which a significant increase in  $Rn$  above a baseline signal (horizontal black line) can first be detected. Two replicate plots were performed for each standard sample, but the data for only one are shown here. Bottom: Standard curve plotting log starting copy number vs.  $C_t$  (threshold cycle). The black dots represent the data for standard samples plotted in duplicate and the red dots the data for unknown genomic DNA samples plotted in triplicate. The standard curve shows 4 orders of linear dynamic range.

samples. We selected these 2 genes because they are located in 2 chromosome regions (*app*, 21q21.2; *alb*, 4q11-q13) in which no obvious genetic changes (including gains or losses) have been observed in breast cancers (Kallioniemi *et al.*, 1994). The ratio for the 18 normal leukocyte DNA samples fell between 0.7 and 1.3 (mean  $1.02 \pm 0.21$ ), and was similar for the 108 primary breast-tumor DNA samples (0.6 to 1.6, mean  $1.06 \pm 0.25$ ), confirming that *alb* and *app* are appropriate reference disomic genes for breast-tumor DNA. The low range of the ratios also confirmed that the nucleotide sequences chosen for the primers and probes were not polymorphic, as mismatches of their primers or probes with the subject's DNA would have resulted in differential amplification.

#### *myc*, *ccnd1* and *erbB2* gene dose in normal leukocyte DNA

To determine the cut-off point for gene amplification in breast-cancer tissue, 18 normal leukocyte DNA samples were tested for the gene dose (N), calculated as described in "Material and Methods". The N value of these samples ranged from 0.5 to 1.3 (mean  $0.84 \pm 0.22$ ) for *myc*, 0.7 to 1.6 (mean  $1.06 \pm 0.23$ ) for *ccnd1* and 0.6 to 1.3 (mean  $0.91 \pm 0.19$ ) for *erbB2*. Since N values for *myc*, *ccnd1* and *erbB2* in normal leukocyte DNA consistently fell between 0.5 and 1.6, values of 2 or more were considered to represent gene amplification in tumor DNA.

#### *myc*, *ccnd1* and *erbB2* gene dose in breast-tumor DNA

*myc*, *ccnd1* and *erbB2* gene copy numbers in the 108 primary breast tumors are reported in Table I. Extra copies of *ccnd1* were more frequent (23%, 25/108) than extra copies of *erbB2* (15%, 16/108) and *myc* (10%, 11/108), and ranged from 2 to 18.6 for *ccnd1*, 2 to 15.1 for *erbB2*, and only 2 to 4.6 for the *myc* gene. Figure 2 and Table II represent tumors in which the *ccnd1* gene was amplified 16-fold (T145), 6-fold (T133) and non-amplified (T118). The 3 genes were never found to be co-amplified in the same tumor. *erbB2* and *ccnd1* were co-amplified in only 3 cases, *myc* and *ccnd1* in 2 cases and *myc* and *erbB2* in 1 case. This favors the hypothesis that gene amplifications are independent events in breast cancer. Interestingly, 5 tumors showed a decrease of at least 50% in the *erbB2* copy number ( $N < 0.5$ ), suggesting that they bore deletions of the 17q21 region (the site of *erbB2*). No such decrease in copy number was observed with the other 2 proto-oncogenes.

#### Comparison of gene dose determined by real-time quantitative PCR and Southern-blot analysis

Southern-blot analysis of *myc*, *ccnd1* and *erbB2* amplifications had previously been done on the same 108 primary breast tumors. A perfect correlation between the results of real-time PCR and Southern blot was obtained for tumors with high copy numbers ( $N \geq 5$ ). However, there were cases (1 *myc*, 6 *ccnd1* and 4 *erbB2*) in which real-time PCR showed gene amplification whereas Southern-blot did not, but these were mainly cases with low extra copy numbers (N from 2 to 2.9).

#### DISCUSSION

The clinical applications of gene amplification assays are currently limited, but would certainly increase if a simple, standardized and rapid method were perfected. Gene amplification status has been studied mainly by means of Southern blotting, but this method is not sensitive enough to detect low-level gene amplification nor accurate enough to quantify the full range of amplification values. Southern blotting is also time-consuming, uses radioactive

reagents and requires relatively large amounts of high-quality genomic DNA, which means it cannot be used routinely in many laboratories. An amplification step is therefore required to determine the copy number of a given target gene from minimal quantities of tumor DNA (small early-stage tumors, cytopuncture specimens or formalin-fixed, paraffin-embedded tissues).

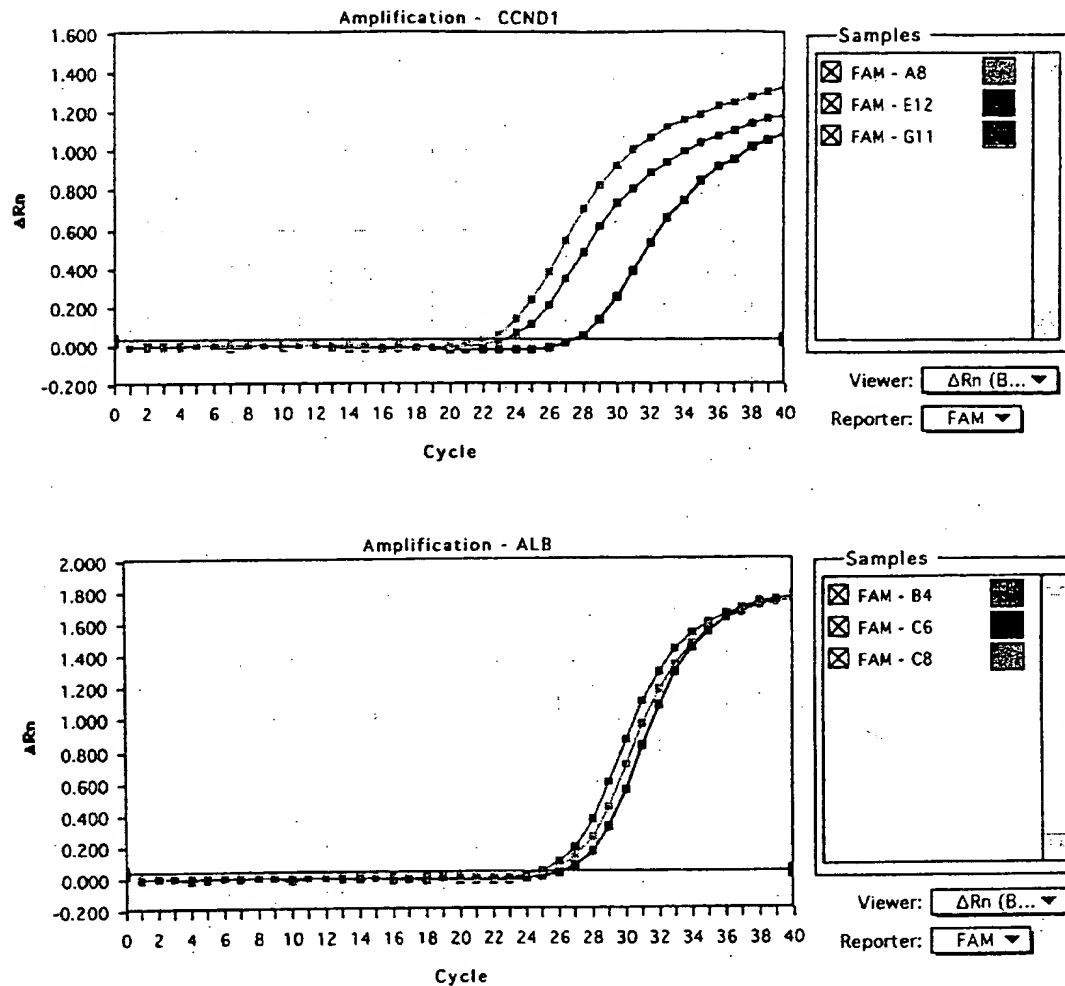
In this study, we validated a PCR method developed for the quantification of gene over-representation in tumors. The method, based on real-time analysis of PCR amplification, has several advantages over other PCR-based quantitative assays such as competitive quantitative PCR (Celi *et al.*, 1994). First, the real-time PCR method is performed in a closed-tube system, avoiding the risk of contamination by amplified products. Re-amplification of carryover PCR products in subsequent experiments can also be prevented by using the enzyme uracil N-glycosylase (UNG) (Longo *et al.*, 1990). The second advantage is the simplicity and rapidity of sample analysis, since no post-PCR manipulations are required. Our results show that the automated method is reliable. We found it possible to determine, in triplicate, the number of copies of a target gene in more than 100 tumors per day. Third, the system has a linear dynamic range of at least 4 orders of magnitude, meaning that samples do not have to contain equal starting amounts of DNA. This technique should therefore be suitable for analyzing formalin-fixed, paraffin-embedded tissues. Fourth, and above all, real-time PCR makes DNA quantification much more precise and reproducible, since it is based on  $C_t$  values rather than end-point measurement of the amount of accumulated PCR product. Indeed, the ABI Prism 7700 Sequence Detection System enables  $C_t$  to be calculated when PCR amplification is still in the exponential phase and when none of the reaction components is rate-limiting. The within-run CV of the  $C_t$  value for calibrator human DNA (5 replicates) was always below 5%, and the between-assay precision in 5 different runs was always below 10% (data not shown). In addition, the use of a standard curve is not absolutely necessary, since the copy number can be determined simply by comparing the  $C_t$  ratio of the target gene with that of reference genes. The results obtained by the 2 methods (with and without a standard curve) are similar in our experiments (data not shown). Moreover, unlike competitive quantitative PCR, real-time PCR does not require an internal control (the design and storage of internal controls and the validation of their amplification efficiency is laborious).

The only potential disadvantage of real-time PCR, like all other PCR-based methods and solid-matrix blotting techniques (Southern blots and dot blots) is that it cannot avoid dilution artifacts inherent in the extraction of DNA from tumor cells contained in heterogeneous tissue specimens. Only FISH and immunohistochemistry can measure alterations on a cell-by-cell basis (Pauletti *et al.*, 1996; Slamon *et al.*, 1989). However, FISH requires expensive equipment and trained personnel and is also time-consuming. Moreover, FISH does not assess gene expression and therefore cannot detect cases in which the gene product is over-expressed in the absence of gene amplification, which will be possible in the future by real-time quantitative RT-PCR. Immunohistochemistry is subject to considerable variations in the hands of different teams, owing to alterations of target proteins during the procedure, the different primary antibodies and fixation methods used and the criteria used to define positive staining.

The results of this study are in agreement with those reported in the literature. (i) Chromosome regions 4q11-q13 and 21q21.2 (which bear *alb* and *app*, respectively) showed no genetic alterations in the breast-cancer samples studied here, in keeping with the results of CGH (Kallioniemi *et al.*, 1994). (ii) We found that amplifications of these 3 oncogenes were independent events, as reported by other teams (Berns *et al.*, 1992; Borg *et al.*, 1992). (iii) The frequency and degree of *myc* amplification in our breast tumor DNA series were lower than those of *ccnd1* and *erbB2* amplification, confirming the findings of Borg *et al.* (1992) and Courjal *et al.* (1997). (iv) The maxima of *ccnd1* and *erbB2* over-representation were 18-fold and 15-fold, also in keeping with earlier results (about

TABLE I - DISTRIBUTION OF AMPLIFICATION LEVEL (N) FOR *myc*, *ccnd1* AND *erbB2* GENES IN 108 HUMAN BREAST TUMORS

Gene	Amplification level (N)			
	<0.5	0.5-1.9	2-4.9	$\geq 5$
<i>myc</i>	0	97 (89.8%)	11 (10.2%)	0
<i>ccnd1</i>	0	83 (76.9%)	17 (15.7%)	8 (7.4%)
<i>erbB2</i>	5 (4.6%)	87 (80.6%)	8 (7.4%)	8 (7.4%)



Tumor	CCND1		ALB	
	$C_t$	Copy number	$C_t$	Copy number
■ T118	27.3	4605	26.5	4365
■ T133	23.2	61659	25.2	10092
■ T145	22.1	125892	25.6	7762

FIGURE 2 - *ccnd1* and *alb* gene dosage by real-time PCR in 3 breast tumor samples: T118 (E12, C6, black squares), T133 (G11, B4, red squares) and T145 (A8, C8, blue squares). Given the  $C_t$  of each sample, the initial copy number is inferred from the standard curve obtained during the same experiment. Triplicate plots were performed for each tumor sample, but the data for only one are shown here. The results are shown in Table II.

30-fold maximum) (Berns *et al.*, 1992; Borg *et al.*, 1992; Courjal *et al.*, 1997). (v) The *erbB2* copy numbers obtained with real-time PCR were in good agreement with data obtained with other quantitative PCR-based assays in terms of the frequency and degree of amplification (An *et al.*, 1995; Deng *et al.*, 1996; Valeron

*et al.*, 1996). Our results also correlate well with those recently published by Gelmini *et al.* (1997), who used the TaqMan system to measure *erbB2* amplification in a small series of breast tumors ( $n = 25$ ), but with an instrument (LS-50B luminescence spectrometer, Perkin-Elmer Applied Biosystems) which only allows end-

TABLE II - EXAMPLES OF *ccnd1* GENE DOSAGE RESULTS FROM 3 BREAST TUMORS<sup>1</sup>

Tumor	<i>ccnd1</i>			<i>alb</i>			<i>Nccnd1/alb</i>
	Copy number	Mean	SD	Copy number	Mean	SD	
T118	4525			4223			
	4605	4603	77	4365	4325	89	1.06
	4678			4387			
T133	59821			9787			
	61659	61100	1111	10092	10137	375	6.03
	61821			10533			
T145	128563			7321			
	125892	125392	3448	7762	7672	316	16.34
	121722			7933			

<sup>1</sup>For each sample, 3 replicate experiments were performed and the mean and the standard deviation (SD) was determined. The level of *ccnd1* gene amplification (*Nccnd1/alb*) is determined by dividing the average *ccnd1* copy number value by the average *alb* copy number value.

point measurement of fluorescence intensity. Here we report *myc* and *ccnd1* gene dosage in breast cancer by means of quantitative PCR. (vi) We found a high degree of concordance between real-time quantitative PCR and Southern blot analysis in terms of gene amplification, especially for samples with high copy numbers ( $\geq 5$ -fold). The slightly higher frequency of gene amplification (especially *ccnd1* and *erbB2*) observed by means of real-time quantitative PCR as compared with Southern-blot analysis may be explained by the higher sensitivity of the former method. However, we cannot rule out the possibility that some tumors with a few extra

gene copies observed in real-time PCR had additional copies of an arm or a whole chromosome (trisomy, tetrasomy or polysomy) rather than true gene amplification. These 2 types of genetic alteration (polysomy and gene amplification) could be easily distinguished in the future by using an additional probe located on the same chromosome arm, but some distance from the target gene. It is noteworthy that high gene copy numbers have the greatest prognostic significance in breast carcinoma (Borg *et al.*, 1992; Slamon *et al.*, 1987).

Finally, this technique can be applied to the detection of gene deletion as well as gene amplification. Indeed, we found a decreased copy number of *erbB2* (but not of the other 2 proto-oncogenes) in several tumors; *erbB2* is located in a chromosome region (17q21) reported to contain both deletions and amplifications in breast cancer (Bièche and Lidereau, 1995).

In conclusion, gene amplification in various cancers can be used as a marker of pre-neoplasia, also for early diagnosis of cancer, staging, prognostication and choice of treatment. Southern blotting is not sufficiently sensitive, and FISH is lengthy and complex. Real-time quantitative PCR overcomes both these limitations, and is a sensitive and accurate method of analyzing large numbers of samples in a short time. It should find a place in routine clinical gene dosage.

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#### REFERENCES

- AN, H.X., NIEDERACHER, D., BECKMANN, M.W., GÖHRING, U.J., SCHARL, A., PICARD, F., VAN ROEYEN, C., SCHNÜRCH, H.G. and BENDER, H.G., *erbB2* gene amplification detected by fluorescent differential polymerase chain reaction in paraffin-embedded breast carcinoma tissues. *Int. J. Cancer (Pred. Oncol.)*, 64, 291-297 (1995).
- BERNS, E.M.J.J., KLIJN, J.G.M., VAN PUTTEN, W.L.J., VAN STAVEREN, I.L., PORTINGEN, H. and FOEKENS, J.A., *c-myc* amplification is a better prognostic factor than *HER2/neu* amplification in primary breast cancer. *Cancer Res.*, 52, 1107-1113 (1992).
- BIÈCHE, I. and LIDEREAU, R., Genetic alterations in breast cancer. *Genes Chrom. Cancer*, 14, 227-251 (1995).
- BORG, A., BALDETORP, B., FERNO, M., OLSSON, H. and SIGURDSSON, H., *c-myc* amplification is an independent prognostic factor in post-menopausal breast cancer. *Int. J. Cancer*, 51, 687-691 (1992).
- CELI, F.S., COHEN, M.M., ANTONARAKIS, S.E., WERTHEIMER, E., ROTH, J. and SHULDNER, A.R., Determination of gene dosage by a quantitative adaptation of the polymerase chain reaction (qd-PCR): rapid detection of deletions and duplications of gene sequences. *Genomics*, 21, 304-310 (1994).
- COURJAL, F., CUNY, M., SIMONY-LAFONTAINE, J., LOUASSON, G., SPEISER, P., ZEILLINGER, R., RODRIGUEZ, C. and THEILLET, C., Mapping of DNA amplifications at 15 chromosomal localizations in 1875 breast tumors: definition of phenotypic groups. *Cancer Res.*, 57, 4360-4367 (1997).
- DENG, G., YU, M., CHEN, L.C., MOORE, D., KURISU, W., KALLIONIEMI, A., WALDMAN, F.M., COLLINS, C. and SMITH, H.S., Amplifications of oncogene *erbB-2* and chromosome 20q in breast cancer determined by differentially competitive polymerase chain reaction. *Breast Cancer Res. Treat.*, 40, 271-281 (1996).
- GELMINI, S., ORLANDO, C., SESTINI, R., VONA, G., PINZANI, P., RUOCCO, L. and PAZZAGLI, M., Quantitative polymerase chain reaction-based homogeneous assay with fluorogenic probes to measure *c-erbB-2* oncogene amplification. *Clin. Chem.*, 43, 752-758 (1997).
- GIBSON, U.E.M., HEID, C.A. and WILLIAMS, P.M., A novel method for real-time quantitative RT-PCR. *Genome Res.*, 6, 995-1001 (1996).
- HEID, C.A., STEVENS, J., LIVAK, K.J. and WILLIAMS, P.M., Real-time quantitative PCR. *Genome Res.*, 6, 986-994 (1996).
- HOLLAND, P.M., ABRAMSON, R.D., WATSON, R. and GELFAND, D.H., Detection of specific polymerase chain reaction product by utilizing the 5' to 3' exonuclease activity of *Thermus aquaticus* DNA polymerase. *Proc. nat. Acad. Sci. (Wash.)*, 88, 7276-7280 (1991).
- KALLIONIEMI, A., KALLIONIEMI, O.P., PIPER, J., TANNER, M., STOKES, T., CHEN, L., SMITH, H.S., PINKEL, D., GRAY, J.W. and WALDMAN, F.M., Detection and mapping of amplified DNA sequences in breast cancer by comparative genomic hybridization. *Proc. nat. Acad. Sci. (Wash.)*, 91, 2156-2160 (1994).
- LEE, I.G., CONNELL, C.R. and BIOCH, W., Allelic discrimination by nick-translation PCR with fluorogenic probe. *Nucleic Acids Res.*, 21, 3761-3766 (1993).
- LONGO, N., BERNINGER, N.S. and HARTLEY, J.L., Use of uracil DNA glycosylase to control carry-over contamination in polymerase chain reactions. *Gene*, 93, 125-128 (1990).
- MUSS, H.B., THOR, A.D., BERRY, D.A., KUTE, T., LIU, E.T., KOERNER, F., CIRINCIONE, C.T., BUDMAN, D.R., WOOD, W.C., BARCOS, M. and HENDERSON, I.C., *c-erbB-2* expression and response to adjuvant therapy in women with node-positive early breast cancer. *New Engl. J. Med.*, 330, 1260-1266 (1994).
- PAULETTI, G., GODOLPHIN, W., PRESS, M.F. and SALMON, D.J., Detection and quantification of *HER-2/neu* gene amplification in human breast cancer archival material using fluorescence *in situ* hybridization. *Oncogene*, 13, 63-72 (1996).
- PIATAK, M., LUK, K.C., WILLIAMS, B. and LIFSON, J.D., Quantitative competitive polymerase chain reaction for accurate quantitation of HIV DNA and RNA species. *Biotechniques*, 14, 70-80 (1993).
- SCHUURING, E., VERHOEVEN, E., VAN TINTEREN, H., PETERSE, J.L., NUNNIK, B., THUNNISSEN, F.B.J.M., DEVILLE, P., CORNELISSE, C.J., VAN DE VIJVER, M.J., MOOI, W.J. and MICHALIDES, R.J.A.M., Amplification of genes within the chromosome 11q13 region is indicative of poor prognosis in patients with operable breast cancer. *Cancer Res.*, 52, 5229-5234 (1992).
- SLAMON, D.J., CLARK, G.M., WONG, S.G., LEVIN, W.S., ULLRICH, A. and MCGUIRE, W.L., Human breast cancer: correlation of relapse and survival with amplification of the *HER-2/neu* oncogene. *Science*, 235, 177-182 (1987).
- SLAMON, D.J., GODOLPHIN, W., JONES, L.A., HOLT, J.A., WONG, S.G., KEITH, D.E., LEVIN, W.J., STUART, S.G., UDOWE, J., ULLRICH, A. and PRESS, M.F., Studies of the *HER-2/neu* proto-oncogene in human breast and ovarian cancer. *Science*, 244, 707-712 (1989).
- VALERON, P.F., CHIRINO, R., FERNANDEZ, L., TORRES, S., NAVARRO, D., AGUIAR, J., CABRERA, J.J., DIAZ-CHICO, B.N. and DIAZ-CHICO, J.C., Validation of a differential PCR and an ELISA procedure in studying *HER-2/neu* status in breast cancer. *Int. J. Cancer*, 65, 129-133 (1996).